Using promoter libraries to reduce metabolic burden due to plasmid-encoded proteins in recombinant *Escherichia coli*

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Abbreviation: **FucA**, L-Fuculose phosphate-aldolase; **SHMT**, serine hydroxymethyl transferase enzyme.

Abstract

The over-expression of proteins in recombinant host cells often requires a significant amount of resources causing an increase in the metabolic load for the host. This results in a variety of physiological responses leading to altered growth parameters, including growth inhibition. Moreover, the expression of other plasmid-encoded genes such as antibiotic resistance genes or repressor proteins may also alter growth.

In this work, we have developed a second generation of an *Escherichia coli* expression system with an antibiotic-free plasmid maintenance mechanism based on an auxotrophic marker (glyA). Metabolic burden related to plasmid maintenance and heterologous protein expression has been minimized by tuning the expression levels of a repressor protein (LacI) and glyA using a selected library of promoters and applying synthetic biology tools that allow the rapid construction of vectors. We apply our engineered antibiotic-free expression

system to the FucA over-production, showing increased production levels.

Our results showed that the aforementioned approaches are of paramount importance in order to increment the protein production in terms of mass and activity.

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Authors’ contributions

MP: Performed all experiments, acquisition and analysis of all the data, as well as in drafting of the manuscript. AFC: Contributed to the conceptual design of the study and manuscript editing. AJ: Involved in the design of constructs and manuscript edition. CdM, GC and PF: Contributed to the overall conceptual design of the study and data interpretation, as well as in drafting and revision of the manuscript. All Authors read and approved the manuscript.

1 Introduction

Among the many systems available, the gram negative bacterium *Escherichia coli* remains one of the most versatile and used host for the production of heterologous proteins, because of its rapid growth rate, the easiness to attain high cell density cultures on inexpensive substrates, its well-characterized genetics and the availability of excellent genetic tools (1). Efforts in developing strategies to maximize the productivity in *E. coli* are well documented in literature (2)(3). Extensive research has been performed over the past years in order to improve recombinant protein production in this cell factory, including the optimization of process parameters such as the growth temperature, the media composition, the induction conditions, as well as engineering the expression system (4).

In particular, thanks to the development of the synthetic biology, new methods and tools to speed up and standardize strain engineering have been established. Compared with conventional DNA cloning protocols, these advanced DNA assembly tools offer an efficient approach to construct multi-gene pathways in a one-step, scar-less, and sequence-independent manner.

Specifically, collections of standard biological parts (BioBricks) allow for the fast assembly of new functions in many organisms (5). For example, individual parts or combinations of parts that encode defined functions can be independently tested and characterized in order to improve the expression system. Besides, parts or devices that do not function as expected can be readily modified or removed(6). DNA construction based on the BioBrick theory has become a key part of most metabolic engineering projects and genetic circuits design. The BioBrick concept exploits the advantage that the same promoters, ribosome binding sites, expression tags, antibiotic resistances and origins of replication are frequently reused, with only the genes of interest being varied (7)(8). Thus, with a series of standardized biological parts, researchers should be able to quickly assemble biological parts (bricks) into different biological functions, pathways and mechanisms.

The recent advances on synthetic biology have allowed to produce novel compounds (9), including high-performance enzymes with applications in the biotechnological sector such as the non ribosomal peptides synthetases (NRPs) and the polyketide synthetases (PKSs) (10)(11). In this context, aldolases constitute one of the most interesting groups of enzymes employed for organic synthesis of carbohydrates, aminoacids and hydroxyacids (12). Aldolases belong to the class of lyases, which catalyze C-C bond formation leading to enantiomerically pure products, even when the starting materials are non-chiral substrates. In particular, L-Fuculose phosphate-aldolase (FucA) catalyzes the reversible reaction of L-fuculose-1-phosphate to dihydroxyacetone phosphate (DHAP) and L-lactaldehyde *in vivo*. *E.*

coli has been proven to be an efficient platform for soluble overexpression of a wide range of aldolases, both endogenous and from other bacteria (13)(14). Initial FucA over expression studies in our laboratory were carried out when the gene *fucA* from *E. coli* K12 was cloned into a plasmid vector controlled by a *Trc* promoter (pTrcfucA) and overexpressed as a recombinant his-tag fusion protein (13). After that, Vidal et al. (15), switched to a commercially available expression system (Qiagen), consisting of the M15 strain and a two-plasmid system including the pQE40 expression vector, which uses the stronger T5 promoter. This system was used to overproduce a range of different aldolases, including a DHAP-dependent aldolase, the rhamnulose 1-phosphate aldolase (RhuA) (16). Moreover, this expression system was further evolved into an auxotrophic marker-based plasmid maintenance system by knocking out the *glyA* gene from the M15 prototrophic strain and by constructing a pQE-40 derived plasmid containing the auxotrophic marker gene (*glyA*) under the control of a weak constitutive promoter (17). The *E. coli glyA* gene encodes for the enzyme serine hydroxymethyl transferase (SHMT). This 46 kDa enzyme has two activities: i) threonine aldolase activity, which catalyzes the reversible interconversion between L-threonine and glycine plus acetaldehyde, and ii) serine hydroxymethyl transferase activity, which catalyzes the reversible interconversion between serine and glycine (18).

Previous studies have shown that the *glyA*-based antibiotic-free system of plasmid maintenance in *E. coli* is a promising alternative approach to the use of antibiotic selection markers. However increased SMHT levels might burden the cell's metabolisms, which lead to a decrease in activity and specific productivity profiles compared to the original system. Furthermore, according to Glick (19), the biosynthesis of proteins is an energy intensive process and it is therefore not surprising that the overproduction of one or more foreign proteins encoded within the DNA may cause segregational instability, where it is often all or part of the foreign gene of interest that is deleted from the plasmid.

Moreover, the presence of a second antibiotic resistance marker in the second plasmid (pREP4), expressing the lacI repressor, impedes the achievement of an expression system completely free from the use of antibiotics. For these reasons, further studies to fine-tune the *glyA* and *lacI* expression levels and to eliminate the pREP4 plasmid are needed in order to overcome these limitations.

In this study, the *fucA* gene has been cloned into the Vidal system in order to obtain high intracellular expression levels. Furthermore, through the application of different synthetic biology approaches, the design and construction of an M15/pQE40-derived expression system consisting of a single vector have been possible. Moreover, the expression levels of the key genes *lacI* and *glyA* have been tuned by the use of different constitutive promoters.

Despite the large diversity of existing DNA shuffling protocols for collecting variant gene libraries (20)(21), the golden gate method based on the type IIs restriction enzymes has been used for assembling the BioBrick vectors. This protocol allows the assembling of multiple DNA fragments into a plasmid by a single restriction-ligation reaction.

In addition, to completely avoid the presence of the antibiotic resistance gene, considered unacceptable in many areas of biotechnology by regulatory authorities (22), the expression system has been further engineered to be finally devoid of antibiotic resistance marker genes and tested for FucA production in shake flasks.

2 Materials and methods

2.1 Bacterial strain

In this work, the K12 derived strains *E. coli* M15 and M15*ΔglyA* have been used and were constructed as previously described (17). The strain *E. coli* DH5α has been used for expression vector engineering purposes, whereas the M15*ΔglyA* strain has been used for recombinant FucA expression. Bacterial strains and plasmids used in this study are summarized in table 1. The strains were stored at -80°C in cryo-stock aliquots prepared from exponential phase cultures grown in Luria-Bertani (LB) medium.

Tabla 1 List of *Escherichia coli* strains and plasmids used

Strains and plasmids	Characteristics	Reference or source
E. coli		
M15	K12 derived	QIAGEN (Villarejo and Zabin 1974; Zamenhof and Villarejo, 1972)

M15 *Δ glyA*	Deletion of the *glyA* locus of the chromosome	(17)
DH5α	Cloning application	Invitrogen
Vectors		
pTrcfuc	pTrc HisC vector with the *fucA* gene cloned	Garcia-junceda et al., 1995 (13)
pQE-FucA	pQE-40 derived (QIAGEN) with *fucA* gene cloned	This study
pREP4	$LacI^q$ kan^R	Qiagen
pQEαβFucA	pQE-FucA derived with fragment, containing the *glyA* gene, under P3 promoter transcriptional control	This study
pSB1C3_GG	High-copy cam^R, used for BioBricks standard assembly vector, carried the *mCherry* gene, with two BsaI restrictions sites with the two different overhangs.	Synth-bio group (group leader A. Jaramillo). Institute of System and Synthetic Biology (iSSB), university of Évry-Val-d'Essonne Genopole® (France)
pSB1C3_GG J231XX-*lacI-glyA*	pSB1C3_GG derived with a library of promoter for *lacI* and *glyA* expression	This study
pQE-FucA_puzzle (J23110)	pQE-FucA derived with insertion of the cassette (J23110-*lacI-glyA*) for *lacI* and *glyA* genes transcription	This study
pQE-FucA_puzzle (J23110) AmpR-	pQE-FucA_puzzle (J23110) derived without the *bla* gene	This study

2.2 Molecular biology techniques

Plasmid and strain constructions. Plasmid DNA and DNA fragments were isolated or purified using PreYield™ plasmid miniprep system and Wizard® SV gel and PCR clean-up system (Promega) according to the manufacturer's instructions. Restriction enzymes were purchased from Thermo Scientific and T4 DNA ligase from Roche. Transformation of *E. coli* DH5α, M15 and M15*ΔglyA* competent cells with the DNA ligation reactions was performed by electroporation using a GenePulser MXcell™ electroporator from Bio-Rad. Transformants were grown on LB-agar medium plates containing the corresponding amount of antibiotic or on defined medium (DM)-agar plates. All the selected clones were confirmed by colony-PCR, single or double restriction digests and DNA sequencing.

PCR reactions. For fragments up to 2.0 Kb, PCR assays were carried out using KOD Polymerase Novagen from Merck Biosciences. For longer DNA sequences, PCR assays were performed using the Phusion high-fidelity DNA polymerase (Thermo Scientific), following the guidelines provided by the manufacturer. For the verification of ligation reactions and transformations, colony PCRs

were performed using the GoTaq® master mix (Promega). All the primers used are listed in Table 2.

Tabla 2 List of primers used in this study. The Tm, melting temperature in °C, and the size in pb are described. Bases in bold represent the BsaI enzyme recognition site; underline bases represent the four different overhangs used for this study; red bases indicate the RBS BBa_B0034 for the *lacI* gene and the green bases specify the RBS J61100 for the *glyA* gene.

Name	Sequence 5'-3'	Tm (°C)	Size (pb)
FucA FW	CGGTACGTGGATCCATGGAACGAAATAAACTTGCTCG	61	37
FucA REW	GCTAGTCCAAGCTTCTCTTCAATTCGTAACCCATAGG	60	37
αβT FW	GCTTGATCTCCGGAGTGAAGACGAAAGGGC	61	31
αβT REW	CGTCCATGTCTAGAGGGCGGATTTGTCCTACTC	63	33
GG_*lacI* FW	TT**GGTCTC**T**TAGC**[2]TCTAGAGAAAGAGGAGAAATACTAGGTGAAACCAGTAACGTTATACG	59	60
GG_*lacI* REW	TT**GGTCTC**T**TCCA**[3]TTCAAGATCTTCACTGCCCGCTTTCC	61	39
GG_*glyA* FW	TT**GGTCTC**T**TGGA**[3]GAAAGAGGGGACAAACTAGTATGTTAAAGCGTGAAATGAA	58	53
GG_*glyA* REW	TT**GGTCTC**T**CGCT**[4]GATAACGTAGAAAGGCTTCC	58	33
PJ1_FW	GCTAAGGATGATTTCTGGAATTC	59	23
PJ2_FW	GCTAAGGCGGACTGCAGGAAGAC	58	23
PJ_REW	TGGCCA CGATAACGTAGAAAGGCTTCC	60	27
J23100_FW	TT**GGTCTC**T**CGCT**[1]TTGACGGCTAGCTCAGTCCTAGGTACAGTGC**TAGC**[2]T**GAGACC**TT		
J23100_REW	AA**GGTCTC**A**GCTA**[2]GCACTGTACCTAGGACTGAGCTAGCCGTCAA**AGCG**[1]A**GAGACC**AA		
J23110_FW	TT**GGTCTC**T**CGCT**[1]TTTACGGCTAGCTCAGTCCTAGGTACAATGC**TAGC**[2]T**GAGACC**TT		
J23110_REW	AA**GGTCTC**A**GCTA**[2]GCATTGTACCTAGGACTGAGCTAGCCGTAAA**AGCG**[1]A**GAGACC**AA		
J23111_FW	TT**GGTCTC**T**CGCT**[1]TTGACGGCTAGCTCAGTCCTAGGTATAGTGC**TAGC**[2]T**GAGACC**TT		
J23111_REW	AA**GGTCTC**A**GCTA**[2]GCACTATACCTAGGACTGAGCTAGCCGTCAA**AGCG**[1]A**GAGACC**AA		
J23117_FW	TT**GGTCTC**T**CGCT**[1]TTGACAGCTAGCTCAGTCCTAGGGATTGTGC**TAGC**[2]T**GAGACC**TT		
J23117_REW	AA**GGTCTC**A**GCTA**[2]GCACAATCCCTAGGACTGAGCTAGCTGTCA**AGAG**[1]C**AGAGACC**AA		

Legend

RBS BBa_B0034 (http://parts.igem.org/Part:BBa_B0034)

RBS BBa_J61100 (http://parts.igem.org/Part:BBa_J61100)

BsaI enzyme recognition site

[1,2,3,4] Four different bp overhang for the BsaI enzyme

The four promoters used (J23100, J23111, J23110 and J23117) in this study were selected from a small combinatorial library of constitutive promoters (Figure 1) (Registry for Standard Biological Parts, http//parts.igem.com). Each promoter was synthetized by oligonucleotide hybridization including two *Bsa*I sites with 2 different overhangs at both, 5' and 3' terminus.

PCR, agarose gel electrophoresis and DNA sequencing were performed to check all the cloning reactions. PCR verification of plasmid constructs was carried out using GoTaq® with the following PCR program: 95°C for 45 s, 55–58°C for 45 s, and 72°C for 0.5–3.5 min, for a total of 30 cycles and a final extension of 72°C for 5 min.

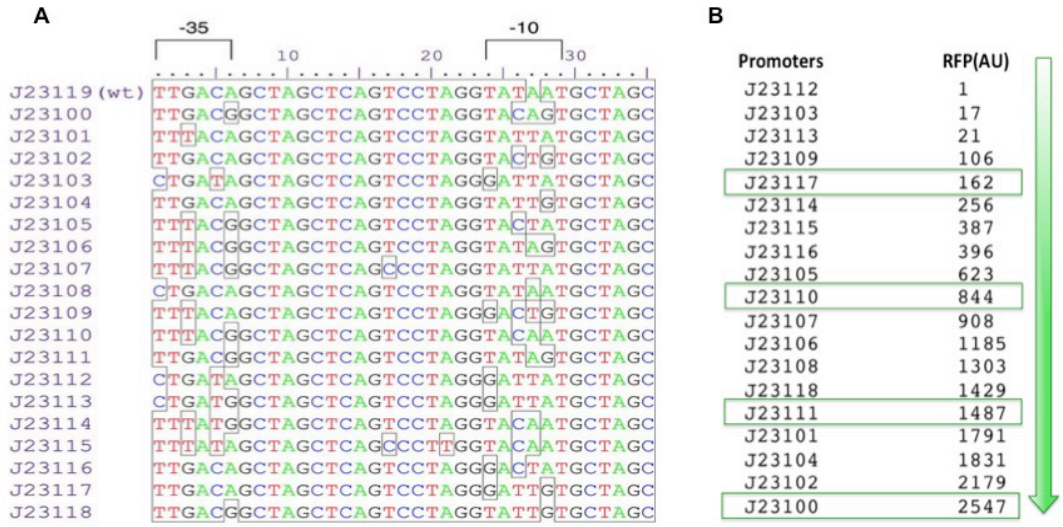

Fig. 1 **A**) The J23119 promoter series is a family of constitutive promoter parts isolated from a small combinatorial library. J23119 is the wild type /wt) promoter sequence and the strongest member of the family. **B**) Reported activities of the promoters are given as the red fluorescence protein (RFP) relative fluorescence (in arbitrary units, AU), pSB1A2 plasmid (Registry for Standard Biological Parts, http//parts.igem.org/parts: pSB1A2) in strain TG1 grown in LB media to saturation. The strength of the promoters goes from the weakest to the strongest, as the orientation of the green arrow on left hand side of the figure. The four promoters used in this study are marked with a green box (J23110, J23111, J23110, J23117).

2.3 Plasmid constructs

pQE-FucA: The commercial vector pQE-40 (Qiagen) was used as reference expression vector for the protein of interest, FucA. This expression vector is based on the IPTG-induced T5 promoter, derived from the T5 phage. This promoter is recognized by *E. coli* RNA polymerase, and has a double *lac* operator (*lac* O) repression module in series to provide tightly regulated and high-level expression of recombinant proteins (Figure. 2A). The *fucA* gene was amplified from the pTrc*fuc* vector using the FucA FW and REW primers (Table 2). Thereafter, the 0.65 Kbp PCR fragment was digested with the restriction enzymes *BamHI* and *HindIII* and subsequently cloned into the pQE-40 vector linearized with the same enzymes, yielding pQE-FucA (Figure. 2B). *E. coli* M15 [pREP4] cells were transformed with the ligation reaction, and the positive clones were selected by growing on LB plates containing of ampicillin and kanamycin.

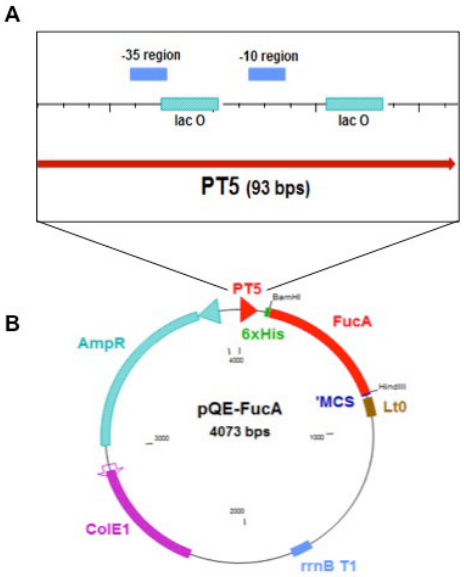

Fig. 2 **A**) T5 promoter region from the pQE-40 expression vector. The *lac O* sequences and the -35 and -10 regions are shown. **B**) pQE-FucA expression vector obtained by cloning the PCR-amplified *fucA* gene into the pQE-40 vector. PT5, promoter T5; 6xHis, histidine affinity tag coding sequence; FucA, *fucA* gene; MCS, multi cloning site; Lt0, lambda t0 transcriptional termination region; rrnBT1, transcriptional termination region; ColE1, replication origin and AmpR, ampicillin resistance gene.

pQEαβFucA: The DNA fragment comprising the *glyA* gene with its own promoter and its 3' termination region (designated as αβTerminator) was amplified using the KOD polymerase and pQEαβRham (17) as template DNA. The resulting 1,784 Kbp fragment was then digested by *BspEI* and *XbaI* and subsequently ligated into the pQE-FucA vector previously linearized with the same enzymes, obtaining the pQEαβFucA. The ligation reaction was transformed into *E. coli* M15Δ*glyA*[pREP4] competent cells and the transformants selected on LB plates containing ampicillin and kanamycin.

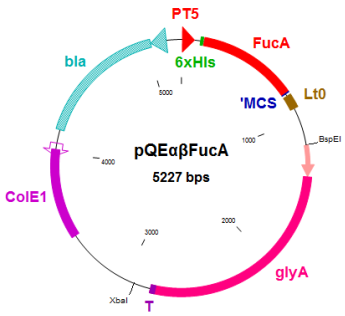

Fig. 3 pQEαβFucA expression vector. PT5, promoter T5; 6xHis, histidine affinity tag coding sequence; FucA, *fucA* gene; 'MCS, multi cloning site; Lt0, lambda t0 transcriptional termination region; *glyA*, glyA gene; T, termination sequence of the *glyA* gene; ColE1, replication origin and AmpR, ampicillin resistance gene.

BioBrick-based vectors (pSB1C3-J231XX_*lacIglyA*).

The BioBrick vectors were assembled using the golden gate technique (23). The Golden Gate method exploit the ability of type IIs endonucleases to cleave DNA outside of the recognition sequence leaving an overhang. In this study *Bsa*I restriction enzyme and its four bp overhangs is used.

The *lacI* and *glyA* genes for the golden gate reaction were amplified from the pREP4 and the pQEαβFucA vectors, respectively with the GG_*lacI* FW and REW primers and with the GG_*glyA* FW and REW primers (Table 2), obtaining the *lacI*_GG and *glyA*_GG modules. For convenience, the starting BioBrick vector pSB1C3_GG, the promoters and the PCR products were diluted to a final concentration of 69 fmol·μL^{-1}. To each reaction, 0.5 µL of *Bsa*I and T4 ligase were added. Final reactions were incubated in a thermocycler as follows: 25-30 cycles, cycling between 37 °C 3 min and 16 °C 4 min, ending with 5 min at 50 °C and 5 min at 80 °C. Four BioBrick constructs were assembled, each one with a different constitutive promoter to tune the expression levels of *lacI* and *glyA* genes. The four vectors were named pSB1C3-J231XX where the double X represents the last two digits of the promoter name. Finally, 2-5 µL of assembly BioBrick constructs were transformed into 50 µL of *E. coli* DH5α competent cells and plated on LB plates containing chloramphenicol. Plasmids were isolated from white colonies and validated as described in section 2.2.

pQE-FucA puzzle (J23110). Constructing new vector starting from the pQE-FucA and the BioBrick vector requires just two-assembly step (Figure 4).

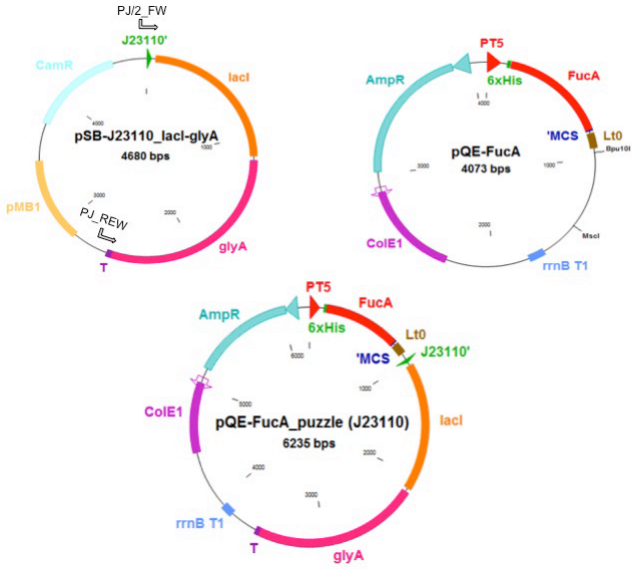

Fig. 4 Strategy for pQE-FucA_puzzle (J23110) construction. A) PCR amplification *lacI-glyA* cassette using plasmid SB1C3-J23110 as template and the primers PJ/2_FW and PJ-REW. B) Double digestion of pQE-FucA with *Bpu10I* and *MscI*. C) Final ligation step of the PCR-amplified fragment (in step A) into the pQE-FucA_puzzle (J23110) expression vector.

The expression cassette J23110-*lacI-glyA* was amplified from the plasmid SB1C3-J23110 by PCR using the Phusion high fidelity polymerase (NEB) and the PJ/2_FW and PJ_REW as amplification primers (Table 2). Both the PCR product and the destination vector pQE-FucA were digested with restriction enzymes *Bpu10I* and *MscI* and subsequently purified after agarose gel electrophoresis. The expression cassette was cloned into the pQE-FucA (double digested) obtaining the pQE-FucA_puzzle (J23110). Finally, the ligation product was transformed into the M15Δ*glyA* competent cells obtaining the M15Δ*glyA* pQE-FucA_puzzle (J23110). Transformants were isolated and correct plasmid sizes were verified.

pQE-FucA_puzzle (J23110)_AmpR$^-$. The vector pQE-FucA_puzzle (J23110) was double digested with *Eco0109I* and *AhdI* in order to eliminate de *bla* gene. The 5´-3´polymerase and the 3´-5´exonuclease activities of the DNA Polymerase I (Large) Klenow Fragment was used in order to end-removal and fill-in terminal unpaired nucleotides.

The blunting DNA reaction, composed of the digested vector about 0.5 mg, 1 µL of dNTPS 25 mM (Bioline) and 1 µL of DNA polymerase I (5U/µL) (NEB), was incubated at room temperature (RT) for 20 min, followed by a heat inactivation step at 75 °C for 10 min. The ligation reaction of the blunt ended DNA fragments and corresponding plasmid backbones was carried out at 16 °C overnight using T4 ligase. The resulting ligation reaction vector was transformed into *E. coli*

M15Δ*glyA* and plated on defined medium (DM) plates. Transformants were isolated and tested both in DM and LB plus ampicillin plates, for positive and negative control, respectively. Transformants were selected as being able to grow in defined media plates but not in LB plates plus ampicillin and validated as described in section 2.2. The best clone in terms of growth and FucA production was selected through a screening process in 500 mL Shake Flasks with 100 mL of defined media for triplicate (data not shown). The induction was performed at the exponential phase with a pulse of 1 mM IPTG and the induction was maintained for 4 hours.

2.4 Culture media

Luria Bertani (LB) medium, containing 10 $g \cdot L^{-1}$ peptone, 5 $g \cdot L^{-1}$ yeast extract and 10 $g \cdot L^{-1}$ NaCl, was used for pre-cultures.

The Defined Medium (DM) used for shake flasks cultures and for the agar plate contained per liter: 5 g glucose, 2.97 g K_2HPO_4, 0.60 g KH_2PO_4, 0.46 g NaCl, 0.75 g $(NH_4)_2SO_4$, 0.11 g $MgSO_4 \cdot 7H_2O$, 0.006 g $FeCl_3$, 0.025 g thiamine, 1.44 g $CaCl_2 \cdot 2H_2O$, 0.07 $mL \cdot 100mL^{-1}$ medium of trace elements solution (TES) (TES contained per liter: 0.04 g $AlCl_3 \cdot 6H_2O$, 1.74 g $ZnSO_4 \cdot 7H_2O$, 0.16 g $CoCl_2 \cdot 6H_2O$, 2.18 g $CuSO_4 \cdot 5H_2O$, 0.01 g H_3BO_3, 1.42 g $MnCl_2 \cdot 6H_2O$, 0.01 g $NiCl_2 \cdot 6H_2O$, 0.23 g $Na_2MoO4 \cdot 5H_2O$).

Stock solutions of kanamycin and chloramphenicol were prepared with a concentration of 100 $mg \cdot mL^{-1}$ and 30 $mg \cdot mL^{-1}$, respectively, and stored at -20 °C. Ampicillin 100 $mg \cdot L^{-1}$ ethanol stock was prepared and stored at -20 °C. IPTG stock was prepared at 100 mM, and stored at -20 °C.

Vitamins, antibiotics, TES, $FeCl_3$, $MgSO_4 \cdot 4H_2O$, $CaCl_2 \cdot 2H_2O$ and inducer were sterilized by filtration (0.2 μm syringe filter made from a blend of cellulose esters, Sartorius). Glucose and saline solutions were separately sterilized by autoclaving at 121°C for 30 min.

2.5 Cultivation conditions

Pre-inoculum. From cryo-stock stored at -80°C, strains were grown in Falcon tubes with 15 mL of LB medium, and supplemented with the corresponding antibiotic (ampicillin and kanamycin final concentration 100 $mg \cdot L^{-1}$, chloramphenicol 30 $mg \cdot L^{-1}$). For the strain pQE-FucA_puzzle (J23110)_$AmpR^-$ no antibiotic was added. Growth was performed overnight at 37 °C with

agitation, and after around 16 h of incubation, cultures reached 2.0-2.5 units of optical density at 600 nm (OD_{600}).

Inoculum. 3 mL of the overnight pre-inoculum were transferred to a shake flask containing 100 mL of DM, with the same growing conditions as pre-inoculum cultures. All cultivations were performed in a working volume of 100 mL in 250 mL-volume baffled shake flasks.

To induce *fucA* expression, an IPTG pulse with a concentration of 1 mM was carried out. Cells were induced when reached an OD_{600} of 1.5 and the induction was maintained for 4 hours, sampling before induction and after 1, 2 and 4 hours of induction.

2.6 Analytical methods

Cell concentration was determined by optical density (OD) measurements at 600 nm using a spectrophotometer (Uvicon 941 Plus, Kontrol). OD values were converted to biomass concentration expressed as Dry Cell Weight (DCW), with 1 OD_{600} equivalent to 0.3 $gDCW \cdot L^{-1}$ (24).

For analysis of substrate and by-products concentration in the cultivation broth, one milliliter of culture medium was separated from biomass by centrifugation at maximum rpm for 6 min and filtered (0.45 μm membrane filter of cellulose esters, Millipore) prior to analysis. Glucose concentration was determined enzymatically on an YSI 2070 system (Yellow Spring System). Acetic acid was analyzed by HPLC (Hewlett Paackard 1050) equipped with an ICSep COREGEL 87H3 ICE-99-9861 (Transgenomic) column and an IR detector (HP 1047), using 6 M H_2SO_4 (pH 2.0) as mobile phase, a flow rate of 0.3 $ml \cdot min^{-1}$, at 40 °C.

The biomass yield, $Y_{X/S}$ was calculated using the next equation:

$$Y_{X/S} = \frac{(DCW_{max} - DCW_0)}{(Glc_0 - Glc)}$$

where DCW_{max} and DCW_0 ($g \cdot L^{-1}$) are the maximum and the initial biomass value, and the Glc_0 and Glc_f ($g \cdot L^{-1}$) are the initial and the final value of glucose concentration, respectively.

The specific substrate uptake rate, q_S, is defined as follows:

$$q_S = \frac{\mu}{Y_{X/S}}$$

where q_S is given as grams of carbon per grams of biomass per hour ($g \cdot g^{-1}DCW \cdot h^{-1}$).

2.7 Enzyme activity for FucA

Samples from culture broths were withdrawn, adjusted to a final OD_{600} of 3, centrifuged and then processed as previously described (25)(17). Briefly, after rejecting the supernatant, the pellets were resuspended in 100 mM TrisHcl (pH 7.5) keeping the same OD600 previously adjusted. Cell suspensions were placed in ice and sonicated with four 15 seconds pulses at 50W with 2 minutes intervals in ice between each pulse, using a Vibracell™ model VC50 (Sonics & Materials). Cellular debris was then removed by centrifugation and the clear supernatant was collected for product analysis.

Two different methods were used to quantify the product in each sample. One was used to calculate the FucA activity while the second one was used to quantify the amount of FucA amongst the rest of intracellular soluble proteins (17). One unit of FucA activity is defined as the amount of enzyme required to convert 1 µmol of fuculose-1-phosphate in DHAP and L-lactaldehyde for minute at 25 °C and pH 7.5.

For all the results, average values were plotted with error bars. The error indicates the confidence interval with a confidential level of 90%.

3 Results

3.1 Comparison of FucA expression between M15[pREP4] and M15[pREP4] Δ*glyA* strain

A first stage performance, the reference FucA expression systems *E. coli* M15[pREP4] pQE-FucA and *E. coli* M15Δ*glyA*[pREP4] pQEαβFucA was assessed in shake flasks cultures in defined media (DM). Heterologous expression was induced in mid- exponential phase cultures using 1mM IPTG in order to compare biomass, FucA and acetate production, as well as glucose consumption profile along time (Figure. 5). As it can be seen in figures 5A and 5B, cell growth slows down after IPTG induction and even starts declining in the M15Δ*glyA* strain. The reference M15 strain presents a slightly higher maximum specific growth rate (μ_{max}) of 0.49 ± 0.01 h^{-1} compared to 0.44 ± 0.01 h^{-1} of the M15Δ*glyA*. This effect may be caused by the increase in the metabolic burden due to expression vector maintenance in the M15Δ*glyA* strain.

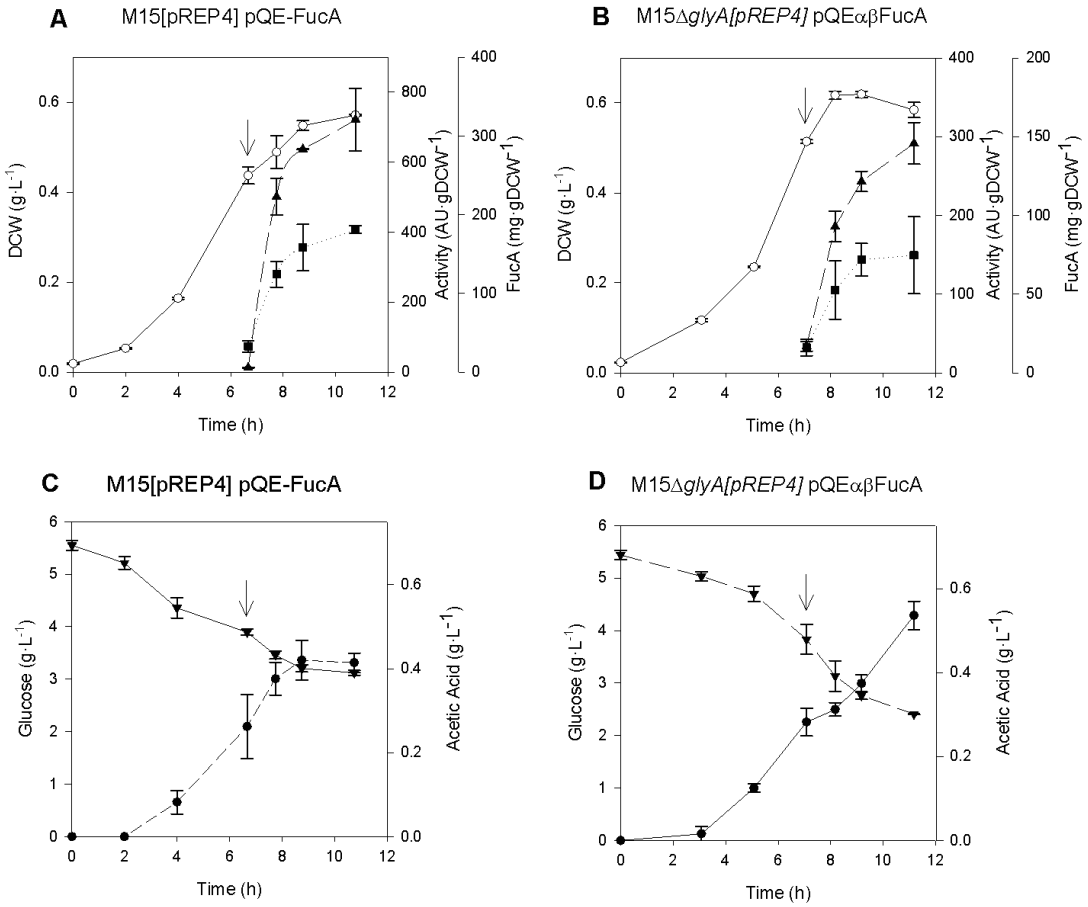

Fig. 5 (O) Biomass DCW (g·L^{-1}),(▲) enzyme activity (AU·$gDCW^{-1}$), (■)specific mass production content (mgFucA·$gDCW^{-1}$), (▼) Glucose (g·L^{-1}) and (●) Acetic Acid (g·L^{-1}) profiles along time in a defined medium. The arrow indicates the IPTG pulse for the induction. A and C refer to the M15[pREP4] pQE-FucA strain while B and D refer to the M15Δ*glyA*[pREP4] pQEαβFucA strain. Shake flasks cultures were performed by triplicate, at 37°C in agitation.

Substrate uptake rates, q_S, along the induction phase for both strains were calculated being 0.37 ± 0.04 and 0.50 ± 0.13 gGlc·g^{-1}DCW·h^{-1} for the M15 and for the M15 Δ*glyA*[pREP4], respectively. As a consequence, the M15 Δ*glyA*[pREP4] strain accumulated higher amounts of acetate throughout the growth process, reaching a final concentration of 0.54 ± 0.03 g·L^{-1} as it can be observed in figure 5D. The physiological background for this acid production under aerobic conditions has been found to be a result of unbalanced rates of glycolysis and the oxidation of the metabolites formed due to saturation of this respiration capacity of E. coli (26). Furthermore, it has been reported, that the recombinant protein production is significantly reduced by acetate accumulation (27). Accordingly, both FucA activity (AU·g^{-1} DCW) and FucA mass (mg·g^{-1}DCW) (Figure 5B) decrease more than 50% comparing the M15Δ*glyA* strain to the reference M15 system (Figure 5A). Whereas the M15 strain reached a final production of 181 ± 5 mgFucA·g^{-1}DCW, with an activity of 721 ± 82 AU·g^{-1}DCW, these values were reduced to 67 ± 37 mgFucA·g^{-1}DCW 291 ± 24 AU·g^{-1}DCW respectively, in the M15Δ*glyA* strain. The results shown above could be related to the concomitant SHMT overexpression observed after induction in the M15Δ*glyA* strain. This fact could impose a slight metabolic burden to the cell, i.e. having a negative impact on the μ_{max} and FucA production.

Noteworthy, the metabolic burden is caused not only due to the overexpression of the protein of interest but also to the expression of other plasmid-encoded genes, e.g. the *glyA* may also contribute (19).

The above statement is in agreement with significant amounts of SHMT accumulated as soluble protein in the cytoplasm of M15Δ*glyA*[pREP4] pQEαβFucA cells, as observed in SDS-PAGE analysis (Figure 6). This suggests that overexpression of the multicopy plasmid-encoded *glyA* gene leads to substantially higher amounts of SHMT, compared to the reference strain containing a single copy of this gene in its genome, thereby imposing a significant burden to the cell's metabolism and affecting negatively the expression of FucA. Overall, these first results pointed at the regulation of the *glyA* expression levels as an important parameter to be taken into account for further improvement of the expression system.

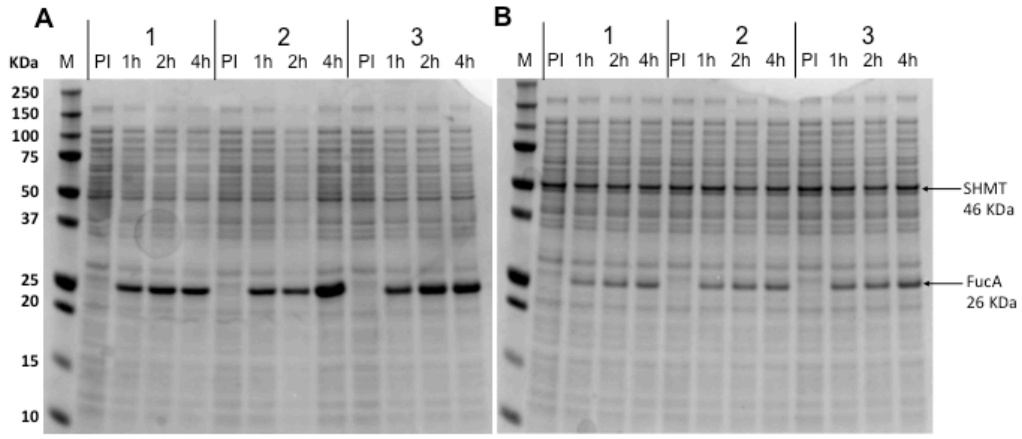

Fig. 6 SDS-PAGE of shake flasks culture's samples. A) Triplicate of the M15[pREP4]/pQE-FucA strain; B) Triplicate of the M15Δ*glyA*[pREP4]/pQEαβFucA strain. Lane M: molecular weight marker. 1, 2, 3 correspond to the shake flask culture replicates while the PI (pre induction) and 1 h, 2h and 4h correspond to the time after induction. The 26 kDa FucA and 46 kDa SHMT bands are indicated in the figure with arrows.

3.2 Single plasmid expression system

The regulation of *glyA* expression levels has a key role in improving the recombinant protein production, as mentioned before. In addition, in order to decrease the extent of the metabolic load and in order to obtain an expression system completely antibiotic free-plasmid selection system, we focused on pREP4 plasmid elimination. The objectives were to i) obtain an expression system based on a single plasmid; ii) clone the *lacI* gene from the pREP4 plasmid to the pQE-expression vector; and iii) tune the *glyA* expression levels.

Accordingly, the pREP4 plasmid was eliminated from the M15Δ*glyA* [pREP4] pQEαβFucA cells, obtaining the new strain M15Δ*glyA* pQEαβFucA. Shake flasks cultures were performed in defined media supplemented with ampicillin (data not shown). An increase in the basal FucA production was expected, due to the removal of the repressor protein encoded by the *lacI* gene present on the pREP4 plasmid. Strikingly, no FucA production was detected in these cultures. To further understand this effect, the *lacI* gene from the pREP4 was amplified and cloned into the pQEαβFucA plasmid, obtaining the pQE-*lacI*-αβFucA expression vector. Again, shake flasks cultures were performed with M15Δ*glyA* strain harboring this plasmid (data not shown), but FucA expression could neither be detected. In order to ensure there was no loss of the expression vector from the cells, the plasmid segregational stability was carried out at different cultivation times before and after induction. Also, M15Δ*glyA* pQE-*lacI*-αβFucA growth on both LB and LB-ampicillin agar plates was tested. Both series of experiments confirmed that the cells maintained the expression vector and, consequently, the lack of FucA expression was not the result of plasmid loss.

A possible explanation for the lack of FucA expression in the system with no *lacI* gene may be related to T5 promoter leakiness in absence of lacI repressor, leading to plasmid structural instability, as discussed in section 3.3. Alternatively, a possible explanation for the lack of FucA expression in the single plasmid system may be that the copy number of the *lacI* gene increases when cloned into the pQE- vector, resulting in significantly higher lacI intracellular levels. In fact, the pQE vector is based on the plasmid ColE1 replication origin, which presents a copy number 2-fold higher compared with the P15A replicon of the pREP4 (1).

Overall, these experiments confirmed the need for tuning the *lacI* transcriptional levels for optimal regulation and induction of the foreign gene expression when re-locating different parts of the reference two-plasmid system into a single plasmid.

3.3 Tuning of *lacI* and *glyA* expression levels

As mentioned in the materials and methods section, the T5 promoter has a double lac O region in order to guarantee a strong repression under non-induction conditions. The *lac* repressor, encoded by the *lacI* gene, binds very tightly to the promoter and ensures efficient repression of the strong T5 promoter interfering with the transcription of the gene of interest. As stated above, promoter leakiness in repressor absence might have lead to structural instability of the expression vector, resulting in reduced expression levels. To test this hypothesis, we sequenced the T5 promoter region isolated from several non-producing M15Δ*glyA* pQEαβFucA constructs. Notably, we observed a deletion in the *lac* O regions, probably due to recombination events of the homology region (data not shown).

This further confirmed the relevance of *lacI* transcriptional levels as key parameter in T5 promoter-based expression systems, influencing both the basal and induced transcriptional levels. For this reason, a series of FucA expressing strains presenting 4 different constitutive transcriptional levels of *lacI* have been constructed as described in the Materials and Methods section (Figure. 1A). A set of 4 calibrated promoters covering a wide range of transcriptional efficiencies has been used. Moreover, because the first generation of the *glyA*-based auxotrophic plasmid maintenance system contained the *glyA* gene under the control of the P3 constitutive promoter, resulting in relatively high amounts of its product, the same set of 4 promoters has also been tested to reduce the transcriptional levels of the *glyA* gene (Vidal L et al., 2008). Thus, an expression cassette was settled where the *lacI* and *glyA* genes have been placed under the control of a constitutive promoter.

3.3.1 pSB1C3 J231XX constructions

In this study, the BioBrick vector pSB1C3 was used as template for the construction of a series of four vectors, each co-expressing the *glyA* and *lacI* genes under the four selected constitutive promoters. That is, each expression vector differs from the other just for the constitutive promoters. The aim was to find the promoter with enough activity to produce the minimum amount of LacI inhibitor preventing "promoter leakiness", as well as the minimal *glyA* transcriptional levels needed for plasmid maintenance and optimal cell growth in defined media. This plasmid series was assembled using a Golden Gate-based strategy. In particular, the pSB1C3 (Figure. 7), a high copy BioBrick assembly plasmid, was prepared by means of a PCR reaction, introducing two *Bsa*I restrictions sites with the two different overhangs. Both the replication origin and the antibiotic resistance marker were maintained as standard parts.

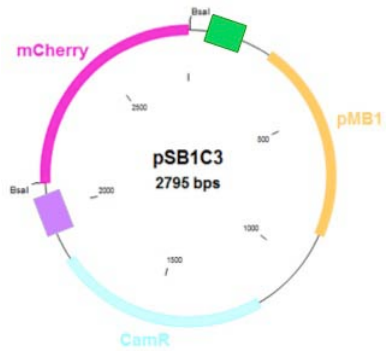

Fig. 7 pSB1C3 vector with the 2 *Bsa*I restriction sites. In pink the *mCherry* gene, in yellow the replication origin (pMB1) and in light blue the chloramphenicol resistance gene (*CamR*).

Both the *lacI* and *glyA* genes were also prepared as PCR products and kept as standard parts of the BioBrick vectors. The *lacI* gene was amplified by PCR from the pREP4 plasmid introducing de novo a strong RBS sequence (BBa_B0034, http://parts.igem.org/Part:BBa_B0034). The DNA part containing the *glyA* gene was obtained by PCR reaction from the pQEαβRham plasmid comprised with a strong RBS sequence (BBa_J61100, http://parts.igem.org/Part:BBa_J61100), i.e. different from the one for *lacI* gene in order to avoid homologous recombination. As terminator sequence we maintained the *glyA* own terminator region, amplified from chromosomal *E. coli* K-12 (17). For both, genes and promoters were introduced at the 5' and 3' terminus the *Bsa*I site with two overhangs (Fig 8A). Remarkably, all golden gate DNA fragments were designed with different overhangs bases in order to have directional cloning and to prevent the relegation of empty vector (Figure. 8B). Thus, reactions could be performed in one-step restriction-ligation, as illustrated in Figure 3B. The final constructs were named pSB1C3-J23XX, where the "X" corresponds to the name of the promoter used (Fig. 8C).

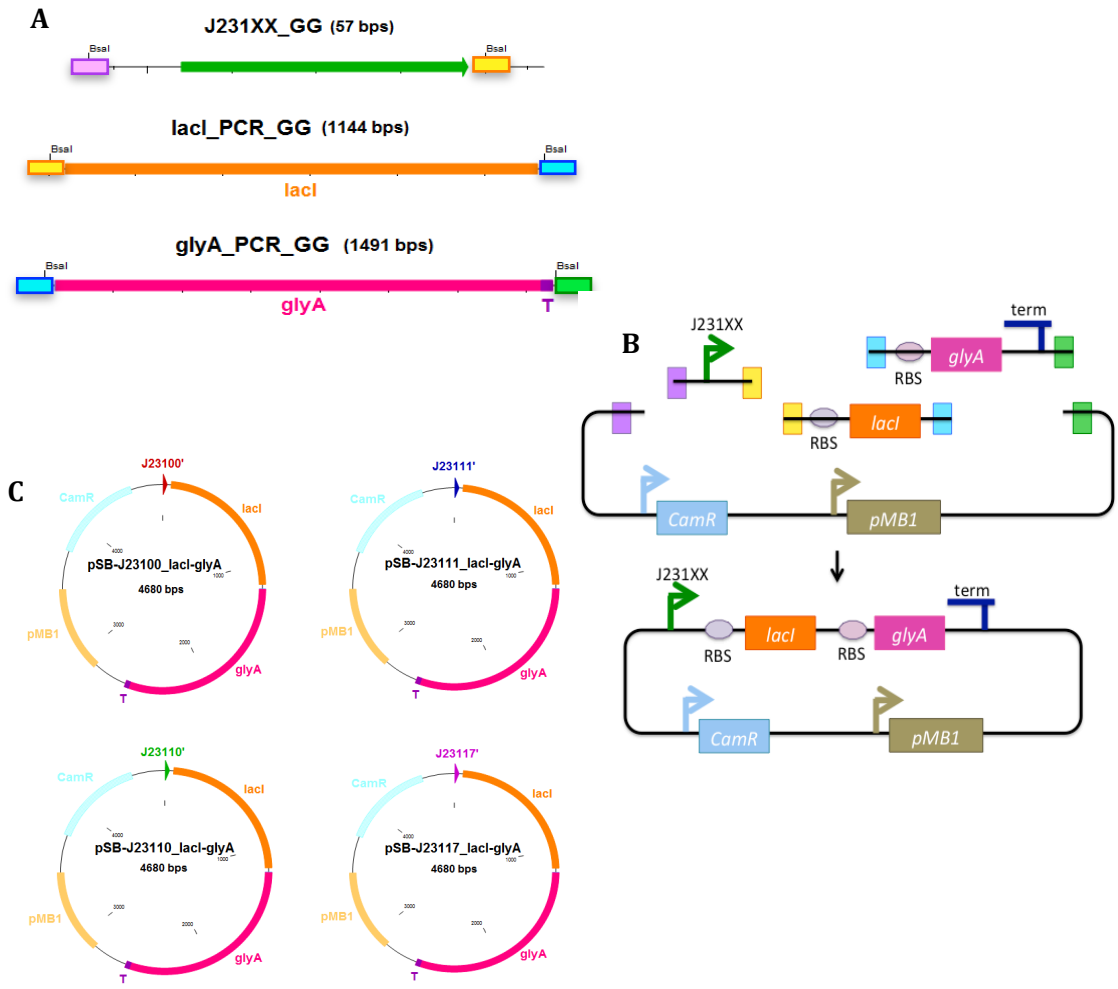

Fig. 8 Golden Gate Assembly Method. A) PCR products of the promoters (J231XX) and the *lacI* and *glyA* genes with the different overhangs of the *Bsa*I at the 5' and 3' terminus in different colors showing the complementary part for the directional cloning. B) Schematic diagram of Golden Gate assembly method to facilitate the construction of the new BioBrick vectors. C) Representation of the four pSB-J231XX vectors, each one with one of the four constitutive promoters.

Once obtained the four expression vectors, they were transformed together with the pQE-FucA plasmid into M15Δ*glyA*. The resulting final strains were named M15Δ*glyA* pQE-40 + pSB1C3-J231XX. To demonstrate the correct assembly of BioBrick parts into the new expression vectors, colony PCR was performed for at least 6 white transformants for each construction. Finally, one correct assembled clone for each strain was selected for further testing in expression experiments in shake flasks cultures.

3.4 Small scale expression experiments with the BioBrick constructs

The over-expression of FucA for the 4 resulting selected transformants was tested in triplicate shake flasks cultures, in which biomass, glucose consumption and enzyme production was analyzed along time (Figure 9). The cultures were grown on 5 $g \cdot L^{-1}$ of initial glucose concentration and when the OD reached 1.5, FucA expression was induced with 1 mM of IPTG. Culture samples were collected before induction and, 1 h and 2h post-induction for further analysis. The evolution of cultures is summarized in figure 9.

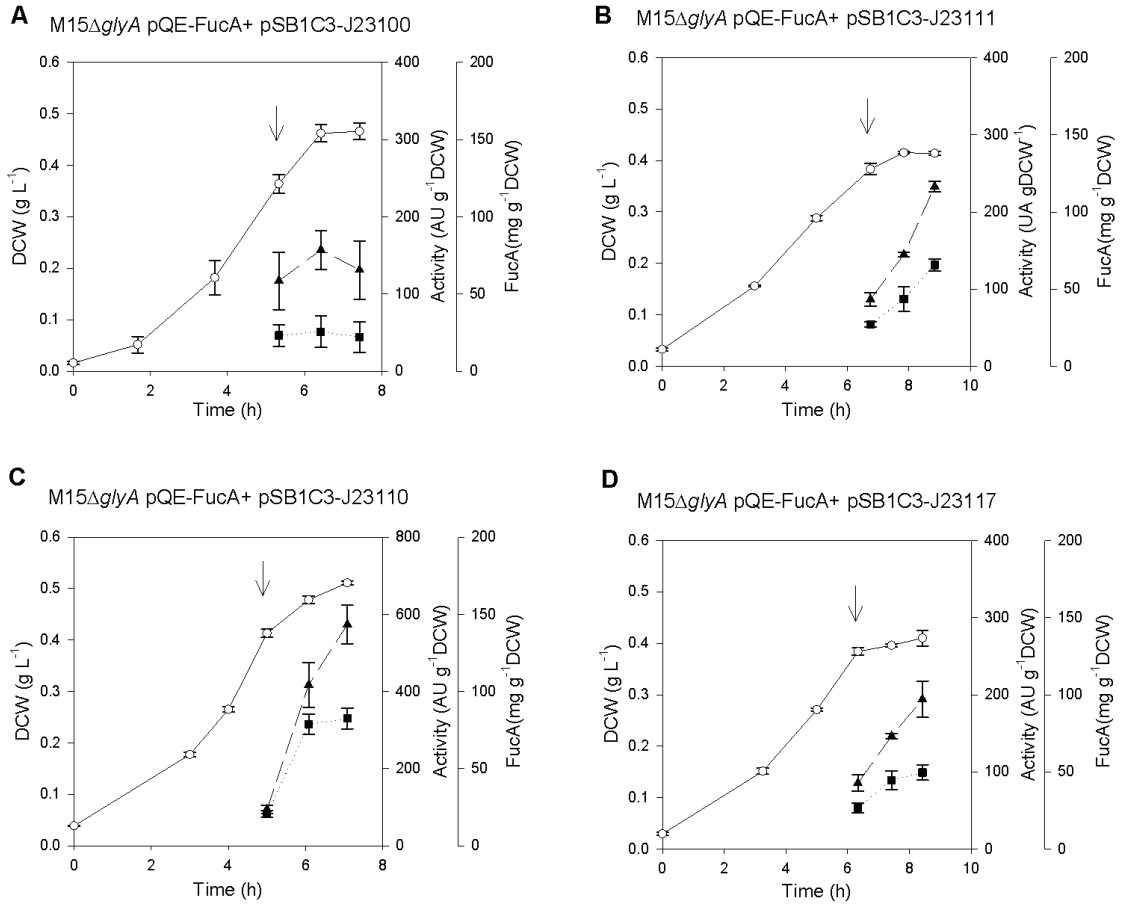

Fig. 9. Shake flask cultures of M15Δ*glyA* pQE-FucA pSB1XX strains series. (O) Biomass DCW($g \cdot L^{-1}$), (▲) enzyme activity ($AU \cdot g^{-1}DCW$) and (■)specific mass production ($mgFucA \cdot g^{-1}DCW$) profiles along time in a defined media shake flasks cultures performed for the 4 different selected transformants, that is, M15Δ*glyA* pQE-FucA pSB1C3 with the 4 different constitutive promoters: A) J23100 B) J23111 C) J23110 D) J23117. The arrow indicates the IPTG pulse for the induction.

The specific growth rate measured in the different cultures showed a similar behavior for all the transformants except for those with the J23100 promoter, which presented a μ_{max} 1.3 fold higher than the reference strain M15 and than the rest of strains (Table 3). Conversely, the use of a stronger promoter for the *lacI* and *glyA* expression such as the J23100 resulted in low detection levels of FucA, both in terms of mass and activity. This observation can be related to

the previous results (Section 3.2). Therefore, a higher growth rate could be explained as follows: higher constitutive *lacI* expression level may lead to a reduction of the *fucA* expression and subsequently to the decrease of the metabolic burden.

Table 3 Maximum specific growth rate (μ_{max} h^{-1}), FucA activity (AU·g^{-1}DCW), FucA mass (mg·g^{-1}DCW), q_S of the induction phase and the maximum acetate yield (g·g^{-1}DCW) for each of the fours selected transformants M15Δ*glyA* pQE-FucA pSB1C3 with the 4 different constitutive promoters: J23100, J23111, J23110 and J23117. The values represent the sample after 2 hour of induction.

Promoter	μ_{max} (h^{-1})	FucA Activity (AU·g^{-1}DCW)	FucA mass (mg·g^{-1}DCW)	q_S (g·g^{-1}DCW·h^{-1})	Acetate yield (g·g^{-1}DCW)
J23100	0.62 ± 0.05	131 ± 35	22 ± 9	0.79 ± 0.10	1.5 ± 0.10
J23111	0.37 ± 0.01	233 ± 7	66 ± 4	0.42 ± 0.09	1.12 ± 0.09
J23110	0.48 ± 0.01	574 ± 49	83 ± 7	0.44 ± 0.06	0.70 ± 0.12
J23117	0.41 ± 0.01	194 ± 24	50 ± 5	0.58 ± 0.01	1.11 ± 0.05

As it can be seen in figure 9C and in table 3, the best expression vector is the one harboring the constitutive promoter J23110, which is in the lower range of tested *lacI* and *glyA* transcriptional levels (Figure 1A). This suggested that the reduced *lacI* and *glyA* genes expression seems to have reduced the energy and building blocks demand for glyA synthesis, as well as minimizing T5 promoter leakiness, resulting in an overall reduced metabolic burden, as reflected in the fact that the μ_{max} of this strain under pre-induction conditions was comparable to that of the M15 reference strain (0.48 ± 0.01 h^{-1}).

Furthermore, when looking at the glucose consumption profile (Fig 10) it can be observed that the 4 different constructs have a similar trend during the exponential phase, while comparing the q_S values during the induction phase it can be clearly seen that the "J23100" strain is the one with higher specific uptake rate of glucose (Table 3). Coherently this strain is the one with the highest yield of acetate reaching 1.50 ± 0.10 gAc·g^{-1}DCW.

Concerning the best expression vector J23110: it is the one that present the lowest qS (0.44 ± 0.06 g·g^{-1}DCW·h^{-1}) and acetate yield (0.70 ± 0.12 g·g^{-1}DCW) comparing with the other constructs.

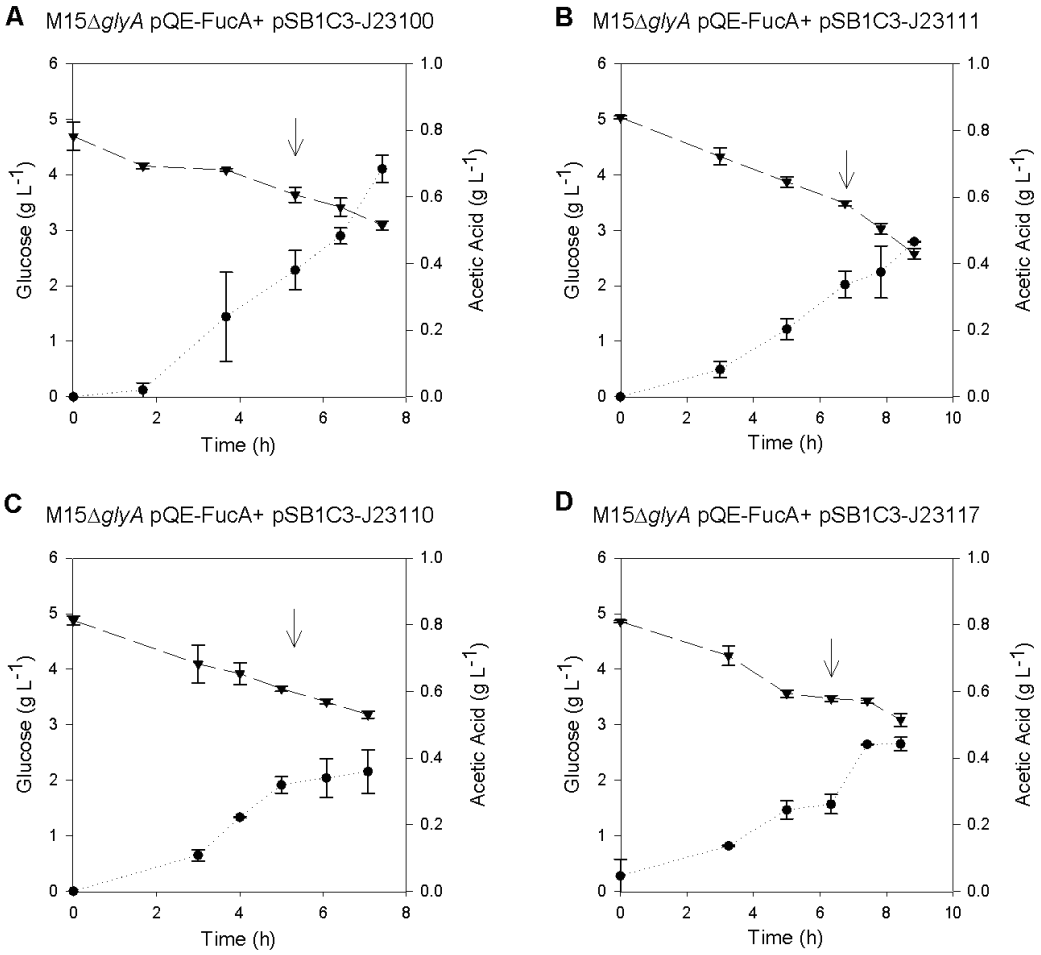

Fig. 10 (▼) Glucose (g·L^{-1}) and (●) Acetic Acid (g·L^{-1}) profiles along time in a defined media shake flasks cultures performed at 37°C 150 rpm for the 4 different selected transformants M15Δ*glyA* pQE-FucA pSB1C3 with the 4 different constitutive promoters: A) J23100 B) J23111 C) J23110 D) J23117. The arrow indicates the IPTG pulse for the induction.

3.5 M15Δ*glyA* pQE-FucA_puzzle strain

Once selected M15Δ*glyA* pQE-FucA + pSB1C3-J23110 as the strain with highest FucA production and specific activity among the 4 different constructs, as well as being able to grow at the same μ_{max} as the reference strain (0.48 ± 0.01 h^{-1}), our next goal was the construction of a single expression vector harboring both the *fucA* gene under control of the inducible T5 promoter and the *lacI-glyA* cassette cloned under the J23110 constitutive promoter. Such plasmid was constructed as described in Materials and Methods section and further transformed into M15Δ*glyA*, yielding *E. coli* M15Δ*glyA* pQE-FucA_puzzle (J23110).

Shake flask cultivations of the new strain were carried out in triplicate at 37°C in defined medium (DM) with 5 g·L^{-1} glucose as initial carbon source, without any antibiotic supplementation. Again, cultures were induced at 1mM of IPTG. The μ_{max} of the M15Δ*glyA* pQE-

FucA_puzzle (J23110) was 0.45 ± 0.01 h^{-1}, comparable to those from the preceding 2-plasmid construct and original reference strain (0.49~0.48 ± 0.01 h^{-1}).

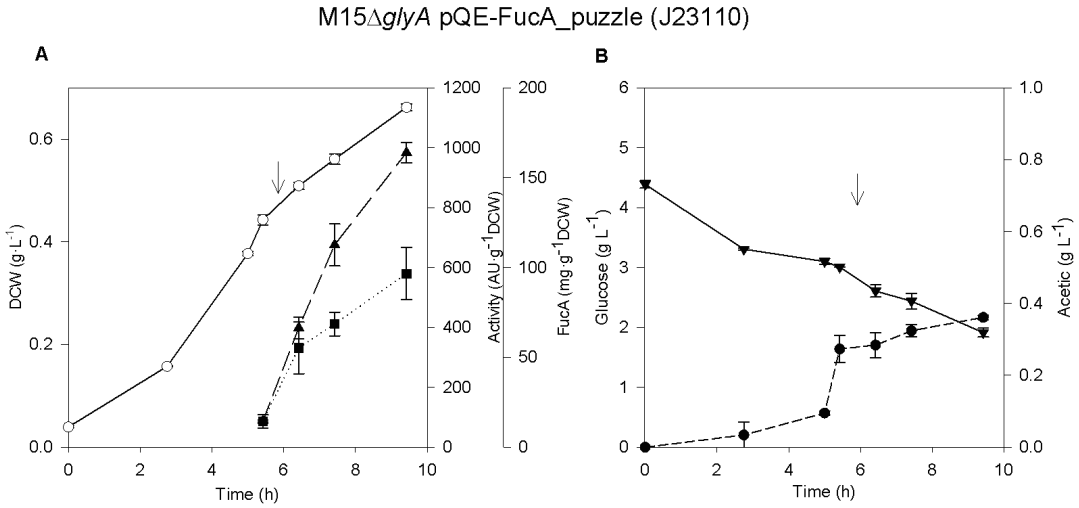

Fig. 11 **A**) (O) Biomass DCW (g·L^{-1}), (▲) enzyme activity (AU·g^{-1}DCW), (■)specific mass content (mgFucA·g^{-1}DCW) and **B**) (▼) Glucose (g·L^{-1}) and (⬬) Acetic Acid (g·L^{-1}) profiles, along time in a defined medium shake flasks cultures performed at 37°C for the M15Δ*glyA* pQE-FucA_puzzle (J23110) strain. The arrow indicates the 1mM IPTG pulse for the induction.

Maximum FucA mass and FucA specific activity reached were 97 ± 14 mg^{-1} FucA·g^{-1}DCW and 984 ± 32 AU·g^{-1}DCW, respectively (Figure 11 A). Comparing these values with those obtained with the M15 pQE-FucA reference strain (181 ± 5 mg FucA·g^{-1} DCW and 721 ± 82 AU·g^{-1} DCW), it can be observed how, even though the amount of the recombinant protein was still lower, the specific activity increased 1.4-fold. Besides, the M15Δ*glyA* pQE-FucA_puzzle (J23110) presented a reduction in the amount of acetate production, being 0.57 ± 0.03 g·g^{-1}DCW, relating to 0.73 ± 0.04 g·g^{-1}DCW of the M15 pQE-FucA reference strain.

Overall, these results suggest that transcriptional tuning of *lacI* expression levels brings to a better *fucA* expression regulation allowing for an extended production formation period, leading to a higher FucA specific activity. Moreover, *glyA* levels also co-down regulated, have a positive effect on the reduction of the metabolic load due to expression of plasmid-encoded genes (also reflected in the reduced acetate production).

These results are in accordance with the observation of Mairhofer et al. (28), who demonstrated that the folding apparatus is severely overstrained in the plasmid-based expression system compared with the plasmid-free cells due to the different transcriptional profile.

3.6 M15Δ*glyA* pQE-FucA_puzzle_Amp^{R-} strain

Lastly, an expression system completely devoid of antibiotic resistance genes was constructed by removing the *bla* gene from the expression vector. The corresponding strain was named *E. coli* M15Δ*glyA* pQE-FucA_puzzle (J23110)_Amp^{R-}. The ampicillin resistance gene (*bla*) was eliminated from the pQE-FucA_puzzle (J23110) plasmid using the Klenow fragment-based blunting DNA technique (Figure. 11).

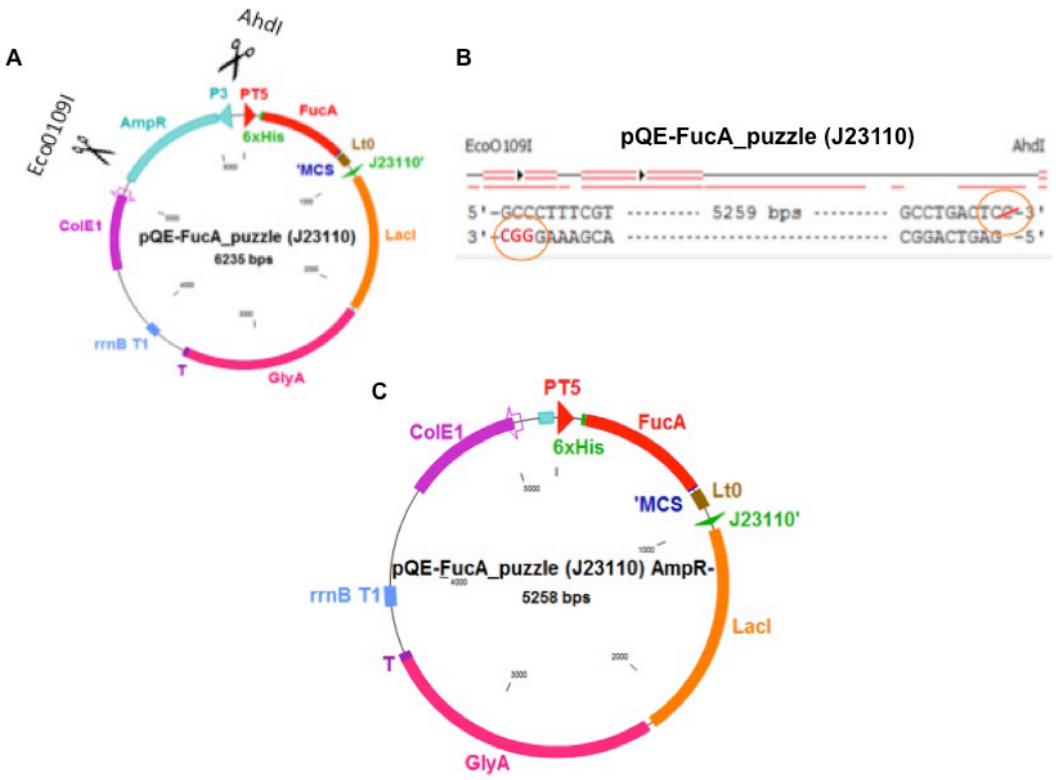

Fig. 12 Schematic diagram of the construction strategy of pQE-FucA_puzzle (J23110)_AmpR−. **A**) Double digestion of pQE-FucA_puzzle (J23110) vector by the restriction enzymes Eco0109I and AhdI. **B**) End removal and fill-in of terminal unpaired nucleotides by DNA polymerase I (large) Klenow fragment (NEB). **C**) Final ligation obtaining pQE-FucA_puzzle (J23110)_AmpR− expression vector.

After a screening process (section 2.3) the best clone in term of best growth rate and higher recombinant protein production was selected (Data not shown).

As for the previous strains, shake flask cultures of the new strain were carried out in triplicate at 37°C in defined medium (DM) without any antibiotic supplementation. The time-profiles of the biomass, glucose consumption, acetate and FucA mass and FucA specific activity were analyzed and represented in figure 14.

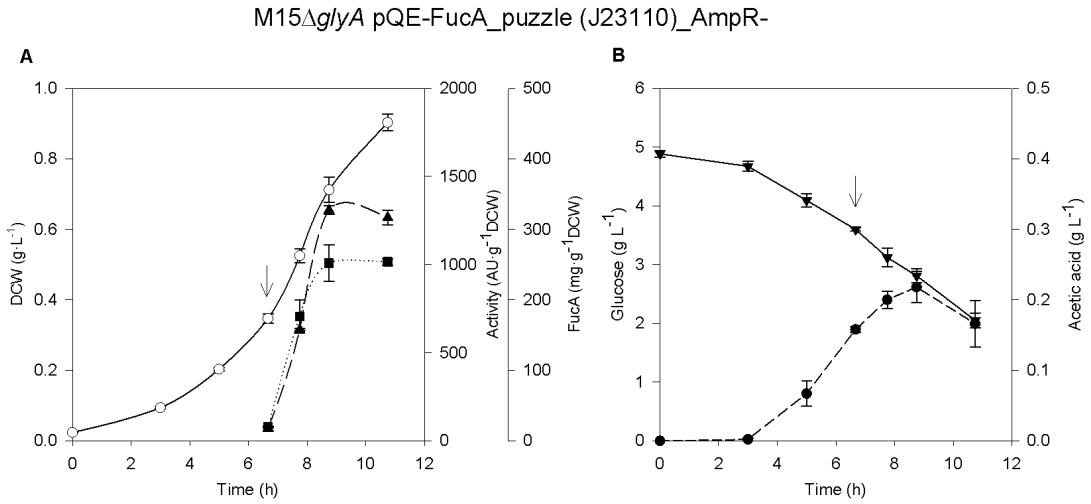

Fig. 13 **A**) (O) Biomass DCW(g·L^{-1}), (▲) enzyme activity (AU·g^{-1}DCW), (■)specific mass (mgFucA·g^{-1}DCW) and **B**) (▼) Glucose (g·L^{-1}) and (⬬) Acetic Acid (g·L^{-1}) profiles, along time in a defined medium shake flasks cultures performed at 37°C 150 rpm for the M15Δ*glyA* pQE-FucA_puzzle (J23110) strain. The arrow indicates the IPTG pulse for the induction.

A small but significant decrease can be observed in the μ_{max} 0.41 ± 0.01 h^{-1} compared to the strains M15[pREP4] pQE-FucA and M15Δ*glyA* pQE-FucA_puzzle (J23110), which showed a μ_{max} of 0.49 ± 0.02 h^{-1} and 0.45 ± 0.01 h^{-1}, respectively (Table 4). However, a significant increase can be observed both for the FucA specific mass and FucA specific activity. The point of maximum activity corresponds to 1309 ± 24 AU·g^{-1}DCW and 254 ± 5 mgFucA·g^{-1}DCW after 2 h of induction. Comparing with all the previous constructs, FucA over-production in the final antibiotic free-plasmid selection system is the highest. As it can be seen in table 4, M15Δ*glyA* pQE-FucA_puzzle (J23110)_Amp^{R-} FucA yield is: i) more than 1.4 fold higher comparing with the M15pQE-FucA strain; ii) two-fold higher comparing with M15Δ*glyA* pQE-FucA + pSB1C3 (J23110) and M15Δ*glyA* pQE-FucA_puzzle (J23110) and iii) three- fold higher referred to the M15Δ*glyA* strain. Coherently, the FucA activity, in terms of AU·g^{-1}DCW, increased through the different strain improvements performed along this work, reaching 4.5-fold higher values when compared to the first Δ*glyA* strain generation. In parallel, the acetate production, expressed as gAc·gDCW^{-1}, was also significantly reduced after these series of expression system engineering steps.

Tabla 4 Maximum FucA activity (AU·g^{-1}DCW), maximum FucA mass (mg·g^{-1}DCW), μ_{max} and maximum acetate yield (g·g^{-1}DCW) along the induction phase for the principal strains presented along this study.

E. coli strains	FucA activity (AU·g^{-1}DCW)	FucA mass (mg·g^{-1}DCW)	μ_{max} (h^{-1})	Acetate yield (g·g^{-1}DCW)
M15[pREP4] pQE-FucA	721 ± 82	181 ± 5	0.49 ± 0.02	0.73 ± 0.04
M15Δ*glyA*[pREP4] pQE-FucA	291 ± 24	67 ± 37	0.44 ± 0.01	0.90 ± 0.04
M15Δ*glyA* pQE-FucA + pSB1C3 J23110	574 ± 49	83 ± 7	0.48 ± 0.02	0.70 ± 0.12
M15Δ*glyA* pQE-FucA_puzzle (J23110)	984 ± 32	97 ± 14	0.45 ± 0.01	0.57 ± 0.03
M15Δ*glyA* pQE-FucA_ puzzle(J23110)_Amp^{R-}	1309 ± 24	254 ± 5	0.41 ± 0.01	0.37 ± 0.01

4 Conclusions

This work has developed a novel expression system based on an antibiotic-free plasmid maintenance mechanism. We demonstrated that a tuning of the glyA and lacI expression was required to reduce metabolic burden. Although the overexpression of the plasmid-encoded protein of interest (FucA) is a major factor in the metabolic burden, the non-optimized expression levels of *lacI* and *glyA* genes, coding for the lac repressor and the auxotrophic selection marker protein respectively, also contributed. The selected expression cassette for the *lacI* and *glyA*, where these genes have been placed under the control of a constitutive promoter J23110, seems to down regulate their transcriptional levels. This tuning, together with the complete deletion of the antibiotic resistance gene, results in to a reduction of the metabolic burden leading to a better stability of expression system that, ultimately, allows an improvement of the recombinant protein production and a reduction of acetate secretion.

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